Fall 10-30-2002

A Study of 5-aminolevulinic Acid and its Methyl Ester Used in In vitro and In vivo Systems of Human Bladder Cancer

Vanaja Manivasager

Paul Wan Sia Heng

Jinsong Hao
Marshall University, haoj@marshall.edu

Wei Zheng

Khee Chee Soo

See next page for additional authors

Recommended Citation

Manivasager, V., Heng, P. W. S., Hao, J., Zheng, W., Soo, K. C., & Olivo, M. (2003). A study of 5-aminolevulinic acid and its methyl ester used in in vitro and in vivo systems of human bladder cancer. International Journal of Oncology, 22(2), 313-318.

Authors
Vanaja Manivasager, Paul Wan Sia Heng, Jinsong Hao, Wei Zheng, Khee Chee Soo, and Malini Olivo

A study of 5-aminolevulinic acid and its methyl ester used in *in vitro* and *in vivo* systems of human bladder cancer

VANAJA MANIVASAGER[1], PAUL WAN SIA HENG[2], JINSONG HAO[2], WEI ZHENG[1], KHEE CHEE SOO[1] and MALINI OLIVO[1]

[1]Division of Medical Sciences, National Cancer Centre, 11 Hospital Drive, Singapore 169610; [2]Department of Pharmacy, National University of Singapore, 18 Science Drive 4, Singapore 117543

Received September 6, 2002; Accepted October 30, 2002

Abstract. The use of 5-aminolevulinic acid and its esters to induce endogenous porphyrins for the purpose of detection of epithelial cancers is being studied extensively in many centres around the world. The challenge is to prepare an efficacious formulation for the purpose of cancer detection. Photodynamic diagnosis of cancer using 5-aminolevulinic acid (ALA) and its ester derivatives is being actively investigated. In this study, we compared ALA with ALA methyl ester (AME) derivative in terms of PpIX fluorescence intensity in *in vitro* and *in vivo* systems of bladder carcinoma. For the *in vivo* system consisting of RT112 xenografts, the modes of drug administration compared were intravenous administration and topical application. The Karl Storz fluorescence endoscopy system was used to obtain macroscopic fluorescence images. The macroscopic images were further analysed for fluorescence intensity distribution. For the intravenous administration, over all time points studied (1, 3, 6 h), AME-PpIX fluorescence was lower than ALA-PpIX fluorescence and was cleared at a faster rate than the ALA-PpIX when administered intravenously. Topical application with two different polymers, Gantrez and Polyvinyl pyrrolidone (PVP) which are fast releasing polymers was found to be comparable in inducing PpIX fluorescence. Topical AME-PpIX fluorescence was found to be comparable with ALA-PpIX fluorescence. The results of this study suggest that the AME can also be used as a good diagnostic agent.

Introduction

Bladder cancer is one of the most prevalent malignant diseases in the world (1). In Singapore, it is the 9th most common type of malignancy. It has been reported that 75-80% of malignancies is superficial in nature and the chances of recurrence after first treatment in these malignancies are 70% (2). Early diagnosis of dysplasia and urothelial tumours plays an important role in prognosis of the disease. The use of endogenous protoporphyrin IX (PpIX) as a photosensitizer to diagnose certain superficial cancers is under extensive study in many centres around the world. This is because it is a non-invasive technique. Endogenous PpIX can be induced by the administration of excess 5-aminolevlinic acid (ALA) or its esterified analogues. ALA is a precursor in the heme biosynthetic pathway of nucleated cells. It is metabolized by certain endogenous enzymes to produce PpIX. PpIX is a photosensitive intermediate by-product in the heme biosynthetic pathway (3). Along with PpIX there are other porphyrin species such as heptaporphyrin, coproprophyrin, and uropophyrin (13,14). Survival of mammalian cells is crucially dependent upon the biosynthesis and metabolism of porphyrins (4). PpIX being the immediate precursor of heme will also be synthesized in equal amounts. However, a tight feedback loop control ensures that only a scarce amount of PpIX is left at any one time (5). Accumulation of the endogenous porphyrins only happens under abnormal conditions such as in malignant or pre-malignant cell transformation. PpIX preferentially accumulates in tumour cells due to changes in the activity of two main enzymes, porphobilinogen deaminase (PBG) and ferrochelatase (FC) (6-8). In tumour cells, while the activity of PBG is increased, the activity of FC is decreased resulting in the build up of PpIX. In normal cells, FC catalyses the conversion of photosensitive PpIX to heme, which is not photosensitive. It chelates the Fe^{2+} into the porphyrin ring structure. The administration of excess exogenous ALA or various esterified analogues of ALA can also induce the excess production of PpIX (topically or intravenously). While ALA is hydrophilic, which is a limitation as it restricts its penetration through cellular membranes; its ester derivatives have been developed to be of increased lipophilicity. In our study we have compared AME with ALA. Increased transmembrane access into the cell, large amounts of ALA can be generated inside the cell due to the abundance of non-specific intracellular esterases in majority of the cells (15). Different esterified analogues behave differently in cells. The cytotoxicity and phototoxicity vary among these esters. Though the esters enter the cell at a faster rate, they need to be cleaved in order to function in the heme biosynthetic pathway. However, the mechanism by which the ALA enters the cells is transported across the membrane via peptide transporters and the transport is electrogenic and coupled to H^+ transport (6,16).

Correspondence to: Dr M. Olivo, Division of Medical Sciences, National Cancer Centre, 11 Hospital Drive, Singapore 169610
E-mail: dmsmcd@nccs.com.sg

Key words: ALA, ALA methyl ester, PpIX, PDD, bladder, topical

PpIX is a well-known photosensitizer that yields sufficient singlet oxygen when activated by light of appropriate wavelength and intensity. ALA is already in use in many centres for photodynamic diagnosis (PDD) and photodynamic therapy (PDT) in various superficial diseases such as actinic keratosis, basal cell carcinoma and squamous cell carcinoma. ALA induced PpIX is also highly fluorescent. Upon activation with blue/violet light, PpIX fluoresces in red. ALA is also used for the detection of neoplastic urothelial lesions, early stage lung cancer, cervical dysplasia and laryngeal neoplasm. Most studies have been done on applications to the skin. It was observed that the skin overlaying the tumours, which looks normal, constitutes a much lower barrier for ALA penetration than the skin overlaying healthy tissues. It was also found that the ALA induces more PpIX fluorescence than certain of its esters do. Systemic uptake is also reported to be higher resulting in high accumulation of PpIX in other vital organs such as the liver, gut and skin. These studies were compared with intravenous or intraperitoneal administrations of ALA (9-12). AME-PDT is currently being studied in basal cell carcinomas in humans. Very few studies have reported the use of AME for cancer detection due to the seemingly low production of PpIX *in vivo*.

One of the aims of this study was to compare the uptake and retention kinetic of ALA and AME in bladder cancer cells *in vitro* and *in vivo*. We went on to conduct this study by using topical applications of ALA and AME and intravenous administration. With the use of macroscopic fluorescence imaging of endogenous PpIX, the optimal selectivity between tumour and normal regions was determined. For the topical application two fast acting polymers Gantrez and Polyvinyl pyrrolidone (PVP) were compared, these are currently being studied for rapid drug delivery and transdermal films. Gantrez is a synthetic copolymer of methylvinyl ether and maleic anhydride. It has several applications such as transdermal films and carriers for mucosal applications of the oral cavity. PVP is also being extensively studied in area of transdermal films (17).

Materials and methods

In vitro cellular uptake studies. RT112 is a grade II (poorly differentiated) human bladder carcinoma cell line (ATCC). It was a kind gift from Clinical Research Centre, National University of Singapore. The cells were grown in culture medium RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 1X L-glutamine, 100 units/ml penicillin and streptomycin (Gibco) and 1X sodium pyruvate in an atmosphere of 5% CO_2 at 37°C. Confluent cells were trypsinized with 0.05% Trypsin-EDTA (Gibco) and seeded onto 100 mm petri dishes ($1.5x10^6$ cells/dish) and left to settle and attach overnight. The culture medium was removed and the cells were washed twice with 1X PBS. The petri dishes were divided into two groups. They were incubated with 15 mM ALA and 15 mM AME in culture medium without serum for 30 min. The cells were then washed with 1X PBS. Fresh medium without serum was added to the dishes and incubated at 37°C with 5% CO_2 for 1, 3, 6, 9 and 24 h. At each time point the cells were washed with 1X PBS, scraped and incubated in perchloric acid and methanol mixture (1:1) for 15 min. ALA and AME induced PpIX was detected using the spectrofluorophotometer RF-5301 PC (Shimadzu). Excitation was set at 400 nm and the emission spectrum range was set at 500 to 700 nm.

Tumour model. RT112 cells were grown in culture medium as for the *in vitro* studies. Once confluent they were washed with 1X phosphate buffered saline and trypsinized with 0.05% Trypsin-EDTA (Gibco). The cells were then re-suspended in 1X Hanks balanced salt solution. Male Balb/c nude mice, 6-8 weeks old, about 20-25 g were obtained from Animal Resource Centre of Australia. The trypsinized cells ($1.5x10^6$) were implanted subcutaneously into the flanks of the nude mice. The mice were housed in micro-isolator cages (5 per cage) fed with filtered air through air vents. Their food, water and bedding were sterilized. The tumour was allowed to grow over the next 7-10 days to a size of 5-8 mm in diameter.

ALA/AME topical applications. For the Gantrez topical application, ALA/AME were dissolved in poly (methyl vinyl) ether/maleic anhydride (Gantrez, ISP, USA)/poly (meth) acrylates (Eudragit, Rohm Pharma, Germany)/alcohol solution. For the PVP, ALA and AME were dissolved in polyvinyl-pyrrolidone (Plasdone, ISP, USA)/alcohol solution. A concentration of 2.5 mg/cm^2 of ALA and AME for both formulations of topical application was prepared. The solution was then poured onto a calculated area of polythene sheets and air dried to form films. The films were cut into 1.5 cm^2 pieces and sealed in bags and stored for further use. Both formulations have muco-adhesive properties.

Animal experiments. Six groups of balb/c nude mice were used. Group 1 was used for the intravenous administration of ALA. Group 2 was used for intravenous administration of AME. Group 3 was used for Gantrez ALA topical application. Group 4 was used for Gantrez AME topical application. Group 5 was used for PVP ALA topical application. Finally group 6 was used for (PVP) AME topical application. For topical application, 5 mice per group were used. The nude mice were anaesthetized with a cocktail of Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml flaunisone, Janssen), Dormicum (5 mg/ml midazolam HCl, David Bull Laboratories) and deionised water (1:1:2). The skin overlaying the tumour was carefully removed to expose the tumour. Extreme care was taken to minimize bleeding as this can interfere with the imaging procedure. A white light image of the tumour was obtained using the Karl Storz fluorescence endoscopy system (FES). This is to help later in identifying the tumour and its margins when comparing with the fluorescence image. The films were then applied on the tumour making sure that the patch covered the tumour well and also part of the normal tissue surrounding the tumour. For the muco-adhesive films the patch was applied for 30 min and then removed. The area was rinsed well with 70% alcohol to remove any residual ALA. The tumour was imaged at different time points, post topical application, by the FES using filtered blue light at 460 nm.

Intravenous administration. ALA/AME was dissolved in deionized water and the pH was adjusted to 6.5. The mice were administered with a dose of 250 mg/kg-body weight by

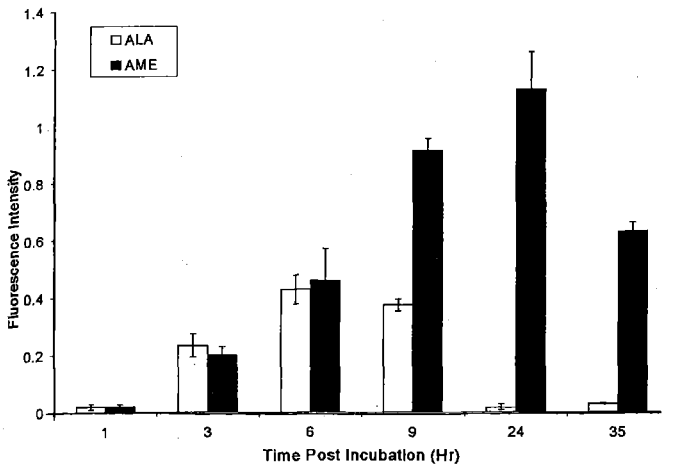

Figure 1. PpIX fluorescence of ALA and AME treated RT112 cells.

tail vein injections. Five animals were used for each time point. They were then kept in darkness. The mice were imaged at various time points, such as 1, 3 and 6 h. The skin overlaying the tumour was removed and the tumours were imaged using the FES. The mice were then sacrificed, the tumours were removed and snap frozen in liquid nitrogen and stored at -80°C for further analysis.

Instrumentation. The system consists of a fluorescence detection unit, an illumination console, a video displaying and recording unit, and a computing system for image acquisition, display and processing. A 100 W xenon arc lamp (D-Light AF system, Karl Storz, Germany) is used for the white light illumination and the PpIX fluorescence excitation when filtered by a band pass filter (370-450 nm). The excitation power of the blue light at the endoscope tip is approximately 50 mw. Both of the light illumination and the observation of the tissue targeted are achieved via a modified endoscope equipped with a long pass (LP) filter (cut-off wavelength at 470 nm). The white light and ALA fluorescence imaging were achieved by a 3-chip colour CCD video camera (Tricam SL_PDD, Karl Storz, Germany) connected to the modified endoscope.

Image analysis. The images thus obtained were analyzed using the image analysis software, MicroImage 4.0. Images obtained in blue light needed to be further processed to obtain normalized fluorescence intensity images. The processing involves contrast enhancement of the original image. This is followed by hue extraction where one extracts the fluorescence colour (red) that is needed. Following hue extraction, the thresholding and segmentation of the area of interest (AOI), which is the tumour in this case, was carried out. This gives us the red-black image. From this we obtained the relative intensity distribution over the tumour and the normal regions. Finally, the normalised intensity image, which is a ratio of the tumour fluorescence to the normal (blue) background, was obtained.

Statistical analysis. Statistical analysis was done using the SPSS 10.0 statistical package for Windows. Analysis was

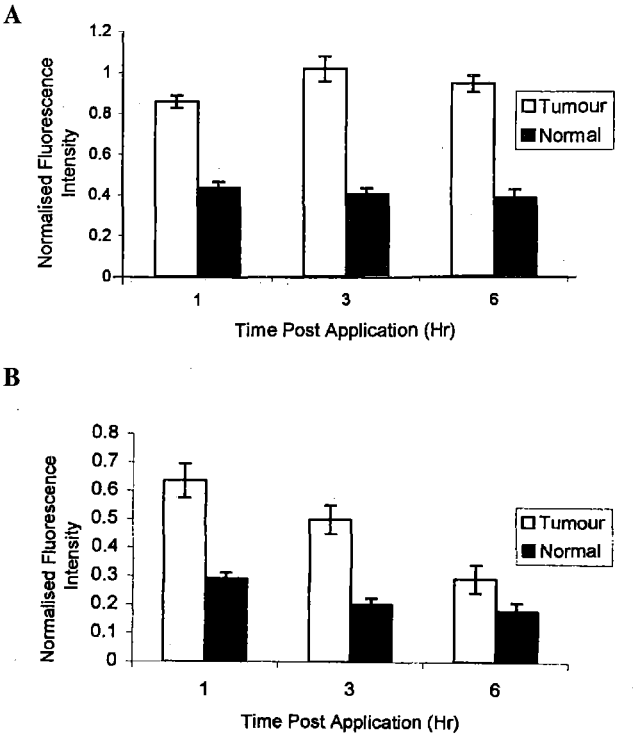

Figure 2. (A), Normalized fluorescence intensity of tumour vs. normal as a function of time for IV ALA. (B), Normalized fluorescence intensity of tumour vs. normal as a function of time for IV AME.

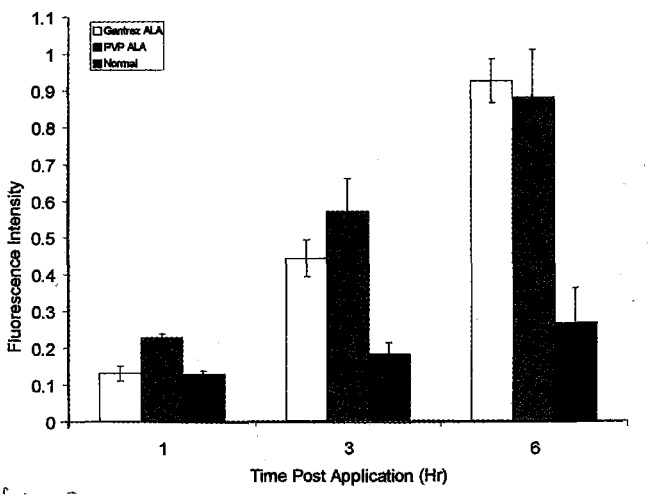

Figure 3. ALA induced PpIX fluorescence for Gantrez and PVP topical application.

done by comparing means (ANOVA). The mean difference was found to be statistically significant ($p<0.05$).

Results

Using the spectrofluorophotometer, we measured the fluorescence intensity of ALA and AME induced PpIX in cells over time. Fig. 1 shows the ALA and AME induced PpIX fluorescence intensity of RT112 cells over time *in vitro*. While ALA induced PpIX fluorescence decreased after 9 h

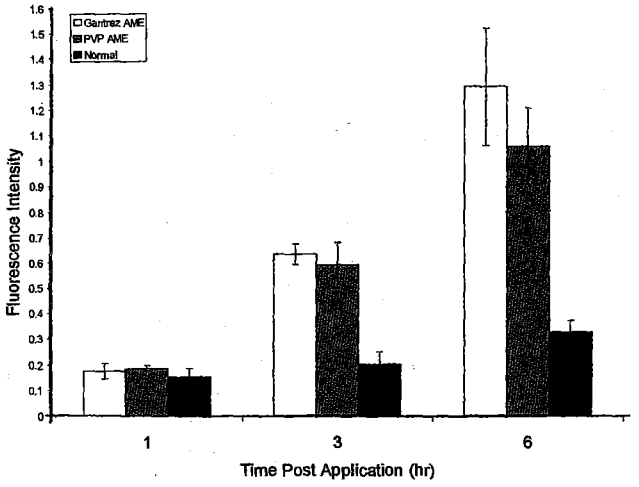

Figure 4. AME induced PpIX fluorescence for Gantrez and PVP topical application.

due to the PpIX being converted to heme, AME induced PpIX fluorescence continued to increase and peaked at around 24 h. This could be due to the fact that AME has to be cleaved before it enters the heme synthesis pathway. Fig. 2 shows the PpIX fluorescence intensity of RT112 tumours when administered with intravenous ALA and AME 250 mg/kg respectively. It was observed that the tumour to normal differential was significantly high from the earliest time point 1 to 6 h. On the other hand AME induced PpIX fluorescence in the tumour was found to be high at 1 h but started to decline thereafter. By 6 h the tumour to normal differential was low. Fig. 3 shows the PpIX fluorescence intensity of RT112 tumours when Gantrez ALA and PVP ALA were applied and imaged at the different time points. It was observed that the tumour to normal differential was increased significantly by 3 h and continued to increase even at 6 h. Fig. 4 shows the PpIX fluorescence intensity of RT112 tumours when Gantrez AME and PVP AME were applied and imaged at the different time points. This pattern was found to be similar to that of topical ALA applications. The only difference was that the AME induced PpIX fluorescence intensity seemed to be slightly higher especially at 6 h. Tumour to normal fluorescence ratio (T/N ratio) was calculated to better appreciate the fluorescence pattern found in the IV administration and the topical applications of the two drugs. Since both topical applications followed the same pattern, only one was chosen to be compared. The T/N ratio of topical PVP ALA and AME induced PpIX increased as a function of time between 1 to 6 h as seen in Fig. 5A. However, the T/N ratio of IV AME induced PpIX fluorescence decreased as a function of time between 1 to 6 h while the IV ALA induced PpIX fluorescence increased as seen in Fig. 5B. Fig. 6 shows a series of fluorescence images of different mice taken using the FES at different time points. The fluorescence pattern for IV and topical application is clearly visible. Fig. 6A-C denotes ALA IV; Fig. 6D and E denotes AME IV; Fig. 6G and H denotes PVP ALA; and Fig. 6J-L denotes PVP AME.

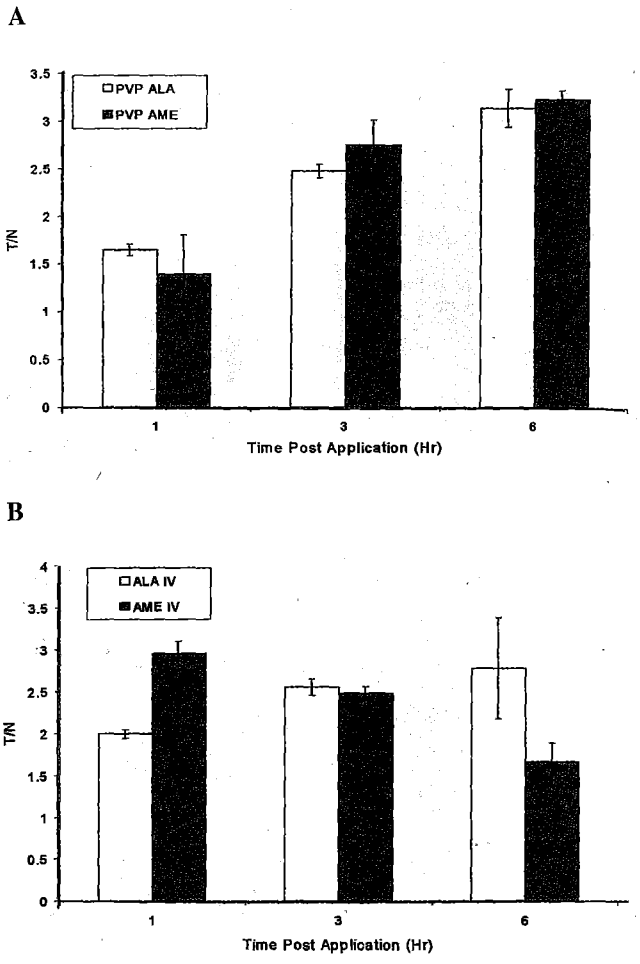

Figure 5. (A), T/N fluorescence ratio of ALA and AME PVP induced PpIX. (B), T/N fluorescence ratio of ALA and AME IV induced PpIX.

Discussion

This study was done to investigate uptake and retention pharmacokinetics of ALA and AME in bladder cells *in vitro*. This pharmacokinetic profile *in vitro* was used to better understand the difference between the *in vitro* and *in vivo*. Subsequently this tumour cell line was xenografted in nude mice and the uptake kinetics studied *in vivo*. Then we went on to compare the modes of administration of the drug. Intravenous administration of ALA for endogenous porphyrin based PDD and PDT, has been the normal mode of administration. AME is still being studied in many centres based on the rationale that esters are transported into cells at a faster rate. However, there are no *in vivo* studies of the intravenous administration of AME in bladder cancer. In our investigations, we removed the skin overlaying the tumour to mimic epithelial condition where the drug does not have to travel through the stratum corneum, sebacious glands, dermal papillae etc. We detected that the intravenous ALA and AME administration not only gives high fluorescence of tumour but also that of normal distal organs such as the skin, normal liver and intestines from as early as 1 h post ALA and AME administration, which is due to considerable amounts of circulating ALA inducing local PpIX. However, it was found that the fluorescence intensity of tumour was reasonably high

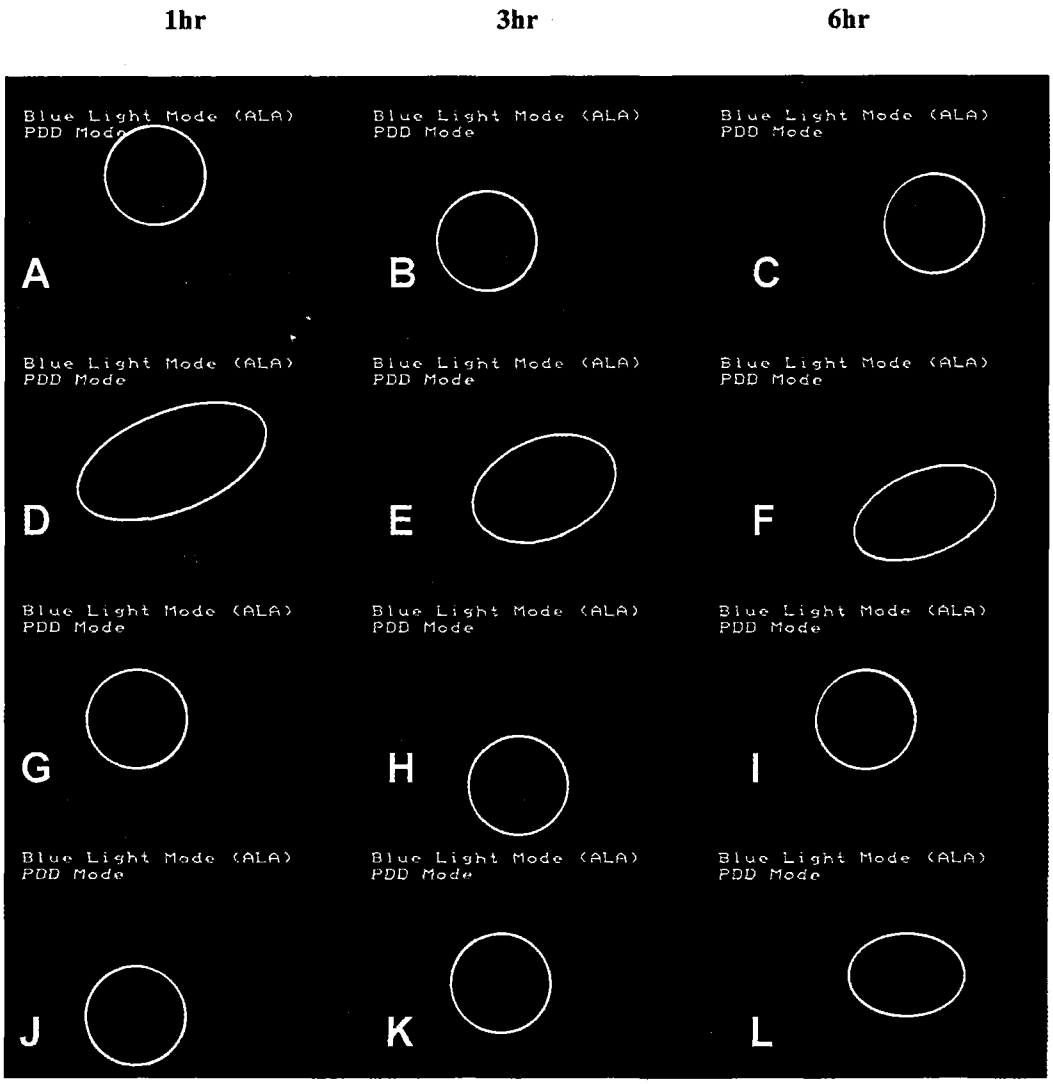

Figure 6. Fluorescence images captured with the FES. (A-C), ALA IV; (D-F), AME IV; (G-I), PVP ALA application; (J-L), PVP AME application. The tumour has been imaged with the surrounding tissue and skin.

from 1 to 6 h post intravenous administration compared to adjacent normal tissue. On the contrary, the topical applications did not produce high fluorescence for either the tumour or normal at 1 h post application. Significant difference between tumour and normal was evident only at the 3-h time point. It is noteworthy that the fluorescence of normal tissue was lower than that of the normal tissue in intravenous application at the same time point. At 6 h the difference between tumour and normal is 4.4 times greater and statistically significant ($p<0.05$). AME behaves differently in the *in vitro* system. PpIX induced by it in the cells is far more than produced by ALA in the cells. This is not the case in the *in vivo* animal study that received the AME IV. The difference observed in the *in vitro* and the IV *in vivo* systems may be attributed to drug metabolism in the *in vivo* system that reduced the drug availability of the parent compound AME to produce PpIX. However, in an *in vivo* system with the topical application of AME (which mimics the *in vitro* system) allows for the longer retention of drug, which is bound to the cell membrane. This discrepancy has been described in other studies (15,18,19). These studies suggest that ALA derivatives with many carbon atoms which are highly lipid soluble might not be converted to ALA by enzymes because such ALA would accumulate at the cell membranes and not diffuse into the cell. The properties of ALA would determine which ALA derivative induces high level of PpIX. The other variable that has an effect on the PpIX production is the availability of oxygen. In *in vitro* studies the oxygen pressure is sufficient for the conversion of ALA derivatives into PpIX. Whereas in an *in vivo* system where the oxygen tension is low, the build up of PpIX is expected to be lower (19). When adjacent skin was analysed for PpIX fluorescence using the topical application, it was found that the AME induced less cutaneous sensitivity when compared to ALA.

Thus, there are merits for ALA and AME delivery via topical applications. This delivery system may take many forms such as rinses, gels or creams, which have to be constituted extemporaneously when needed due to instability of ALA. Films prepared for this study can be made and stored in an airtight container at room temperature for 3 to 6 months. These topical applications also have muco-adhesive properties and can be good candidates for use in the oral cavity PDD and PDT. However, oral rinses require the patient to continuously rinse for 20 min. Hence oral rinses

are not practical for routine clinical use. Also the contact time for ALA on oral tissue is short with the use of an oral rinse.

Despite the expected differences in the availability of ALA in the Gantrez application and PVP application, their *in vivo* performances did not show significant differences. This indicates that the rate-limiting step of ALA delivery to the tumour tissue was the transdermal transport of the hydrophilic ALA compound. However this seems to be tumour dependent (13).

Our study concludes that the amount of PpIX produced, as a function of time (1-6 h) in the *in vivo* system does not correspond to the *in vitro* system. While topical applications will be good for accessible tumours, as the T/N differential is significant by 3 h ($p>0.05$), intravenous administration also has its advantages because high tumour to normal differential is achieved at a shorter duration for both ALA and AME. Between the intravenous administration and the topical application, the difference lies in the fact that there is low systemic uptake in the topical application. Therefore the mode of ALA and AME administration will play an important role in the specific application in cancer diagnosis of the bladder.

Acknowledgements

This study was supported by National Medical Research Council, National Cancer Centre and National University of Singapore, Singapore. The authors would like to thank technical support given by Ms. Bhuvana Shridhar and Mr. William Chin Wei Lim.

References

1. Kantor AF, Hartge P and Hoover RN, *et al*: Epidemiological characteristic of squamous cell carcinoma and adenocarcinoma of the bladder. Cancer Res 48: 3853-3855, 1998.
2. Prout GR Jr: Bladder carcinoma and a TNM system of classification. J Urol 117: 583-590, 1987.
3. Gederaas OA, Rasch MH, Berg K, Lagerberg JWM and Dubbleman TMAR: Photodynamically induced effect in colon carcinoma cells (WiDr) by endogenous photosensitizers generated by incubation with 5-aminolevulinic acid. J Photochem Photobiol B 49: 162-170, 1999.
4. Peng Q, Berg K, Moan J, Kongshaug M and Nesland JM: 5-Aminolevulinic acid-based photodynamic therapy: principles and experimental research. Photochem Photobiol 65: 235-251, 1997.
5. Hillermanns P, Weingandt H, Baumgartner R, Diebold J, Xiang W and Stepp H: Photo detection of cervical intraepithelial neoplasia using 5-aminolevulinic acid-induced porphyrin fluorescence. Am Cancer Soc 88: 2275-2282, 2000.
6. Whitaker CJ, Battah SH, Forsythe MJ, Edwards C, Boyle RW and Matthews EK: Photosensitization of pancreatic tumour cells by δ-aminolevulinic acid esters. Anticancer Drug Des 15: 161-170, 2000.
7. Navone NM, Polo CF, Frisardi AL, Andrade NE and Battle AMDC: Heme biosynthesis in human breast cancer-mimetic '*in vitro*' studies and some heme enzymatic activity levels. Int J Biochem 22: 1407-1411, 1990.
8. Van Hillegersberg R, van den Berg JWO, Kort WJ, Terpstra OT and Wilson JHP: Selective accumulation of endogenously produced porphyrins in a liver metastasis model in rats. Gastroenterology 103: 647-651, 1992.
9. Moan J, Ma LW and Iani V: On the pharmacokinetics of topically applied 5-aminolevulinic acid and two of its esters. Int J Cancer 92: 139-143, 2001.
10. Von Beckerath M, Juzenas P, Ma LW, Inai V, Löfgren L and Moan J: The influence of UV exposure on 5-aminolevulinic acid-induced protoporphyrin IX production in skin. Photochem Photobiol 74: 825-828, 2001.
11. Pahernik S, Langer S, Botzlar A, Dellian M and Goetz AE: Tissue distribution and penetration of ALA induced fluorescence in an amelanotic melanoma after topical application. Anticancer Res 21: 59-63, 2001.
12. Thissen MR, De Blois MW, Robinson DJ, De Bruijn HS, Dutrieux RP, Star WM and Neumann HA: PpIX fluorescence kinetics and increased skin damage after intracutaneous injection of 5-aminolevulinic acid and repeated illumination. J Invest Dermatol 118: 239-245, 2002.
13. Peng Q, Warloe T, Moan J, Godal A, Apricena F, Giercksky KE and Nesland JM: Antitumour effect of 5-aminolevulinic acid-mediated photodynamic therapy can be enhanced by the use of a low dose of photofrin in human tumour xenografts. Cancer Res 61: 5824-5832, 2002.
14. Gederaas OA, Berg K and Romslo I: A comparative study of normal and reverse phase high-pressure liquid chromotography for analysis of porphyrins accumulated after 5-aminolevulinic acid treatment of colon adenocarcinoma cells. Cancer Lett 150: 205-213, 2000.
15. Kloek J and Beijersbergeb van Henegouwen GMJ: Prodrugs of 5-aminolevulinic acid for photodynamic therapy. Photochem Photobiol 64: 994-1000, 1996.
16. Ganapathy ME, Brandsch M, Prasad PD, Ganapathy V and Leibach FH: Differential recognition of ß-lactam antibiotics by intestinal and renal peptide transporters, PEPT1 and PEPT2. J Biol Chem 256: 25672-25677, 1995.
17. Rao PR, Ramakrishna S and Diwan PV: Drug release kinetics from polymeric films containing propranolol hydrochloride for transdermal use. Pharm Dev Technol 5: 465-472, 2000.
18. Kloek J, Akkermans W and Beijersbergeb van Henegouwen GMJ: Derivatives of 5-aminolevulinic acid for photodynamic therapy: enzymatic conversion into protoporphyrin. Photochem Photobiol 67: 150-154, 1998.
19. Ninomiya Y, Itoh Y, Tajima S and Ishibashi A: *In vitro* and *in vivo* expression of protoporphyrin IX induced by lipophilic 5-aminolevulinic acid derivatives. J Dermatol Sci 27: 114-120, 2001.